# Regulation of Autotrophic and Heterotrophic Metabolism in Pseudomonas oxalaticus OX1

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# Regulation of Autotrophic and Heterotrophic Metabolism in *Pseudomonas oxalaticus OX1*: Growth on Mixtures of Oxalate and Formate in Continuous Culture

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**Abstract.** *Pseudomonas oxalaticus* was grown in carbon- and energy-limited continuous cultures either with oxalate or formate or with mixtures of these substrates. During growth on the mixtures, simultaneous utilization of the two substrates occurred at all dilution rates tested. Under these conditions oxalate repressed the synthesis of ribulosebisphosphate carboxylase. The degree of this repression was dependent on the dilution rate and the ratio of oxalate and formate in the medium reservoir. At a fixed oxalate/formate ratio repression was greatest at intermediate dilution rates, whereas derepression occurred at both low and high dilution rates. Progressive derepression of ribulosebisphosphate carboxylase synthesis and of autotrophic $CO_2$ fixation at low dilution rates was attributed to the decreasing concentration of intracellular repressor molecule(s), parallel to the decreasing concentration of the growth-limiting substrates in the culture. To account for the derepression at higher dilution rates, it is proposed that the rate of oxalyl-CoA production from oxalate limits the supply of metabolic intermediates and that additional energy and reducing power generated from formate drains the pools of metabolic intermediates sufficiently to lower the intracellular concentration of the repressor(s). During growth of *Pseudomonas oxalaticus* on the "heterotrophic" substrate oxalate alone, at dilution rates below 10% of the maximum specific growth rate, derepression of ribulosebisphosphate carboxylase synthesis and of autotrophic $CO_2$ fixation was observed to a level which was 50% of that observed during growth on formate alone at the same dilution rate. It is concluded that in *Pseudomonas oxalaticus* the synthesis of enzymes involved in autotrophic $CO_2$ fixation via the Calvin cycle is regulated by a repression/derepression mechanism and that the contribution of autotrophic $CO_2$ fixation to the biosynthesis of cell material in this organism is mainly controlled via the synthesis of these enzymes.

**Key words:** *Pseudomonas oxalaticus* OX1 – Formate and oxalate – Mixed substrates – Diauxie – Continuous culture – Calvin cycle – Derepression.

The bacterium *Pseudomonas oxalaticus* is able to grow on a number of organic acids including formate and oxalate. When the organism grows on formate, energy is generated as the formate is oxidized to carbon dioxide while carbon is assimilated autotrophically via the Calvin cycle. In the metabolism of oxalate, energy is generated by its oxidation to carbon dioxide via formate while in the biosynthesis of cell material the carbon-carbon bond present in the substrate is conserved and carbon is assimilated heterotrophically via the glycerate pathway (Quayle, 1961). The organism may therefore be considered a facultative autotroph.

The regulation of the synthesis of Calvin cycle enzymes in facultatively autotrophic microorganisms is not fully understood. When a "heterotrophic" substrate is added to a culture growing autotrophically, the synthesis of autotrophic enzymes is generally repressed, although the extent to which this repression is exerted appears to be dependent on both the organism and the "heterotrophic" substrate involved. For instance, during growth of *P. oxalaticus* in batch cultures on mixtures of formate plus one of a variety of "heterotrophic" substrates (e.g. acetate, lactate, glyoxylate, glycollate) simultaneous utilization of both substrates occurred. In these mixtures formate served as an ancillary energy source and RuBPCase synthesis was partially or totally repressed. The degree of repression

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*Abbreviations.* RuBPCase = ribulosebisphosphate carboxylase; PMS = phenazine methosulphate; DCPIP = 2,6-dichlorophenolindophenol; FDH = formate dehydrogenase; $S_R$ = concentration of growth-limiting substrate in reservoir

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observed was related to the growth rate supported by the "heterotrophic" substrate involved in such a way that repression was more severe on substrates that supported a higher growth rate (Blackmore and Quayle, 1968; Dijkhuizen et al., 1978). Comparable results have been reported for *Alcaligenes eutrophus* (Stukus and DeCicco, 1970) and *Thiobacillus intermedius* (London and Rittenberg, 1966). Interestingly, growth of *P. oxalaticus* on a mixture of oxalate and formate in batch culture resulted in diauxic growth with formate as the substrate of preference (Dijkhuizen et al., 1978). At the moment no definite explanation is available for this observation, although it is considered that either inhibition of the active transport system of oxalate by formate (Dijkhuizen et al., 1977a) or competition by formate for the CoA required in the oxalyl-CoA cycle (Quayle et al., 1961) or both may be involved.

In this paper we report results obtained during studies on the growth of *P. oxalaticus* with mixtures of formate plus oxalate in carbon- and energy-limited continuous cultures. This cultivation method permits an analysis of the regulation of enzyme synthesis under conditions of low substrate concentrations at controlled submaximum growth rates. By using this approach we hoped to clarify the relationship between the growth rate of the organism and the degree of repression of Calvin cycle enzymes. Preliminary reports of this work have been presented elsewhere (Dijkhuizen and Harder, 1978a, b).

## Materials and Methods

### *Organism*

The organism used, *Pseudomonas oxalaticus* OX1, and its maintenance has been described previously (Dijkhuizen and Harder, 1975).

### *Continuous Culture Experiments*

The organism was grown in carbon- and energy-limited continuous cultures, in a fermenter (working volume 1.0 l), as described previously (Dijkhuizen et al., 1977b). Dry weight was determined with a carbon analyzer (Beckman model 915A), connected to an infrared analyzer (Beckman, model 865). $Q_{O_2}$ values, rate of $^{14}CO_2$ fixation by whole cells, enzyme activities, residual substrate concentrations, and protein were measured in culture samples using the methods described by Dijkhuizen et al. (1978). The viability of the culture was measured directly with the microcolony technique described by Postgate (1969).

### *Calculations*

The rate of $CO_2$ fixation necessary to explain the growth rate of *P. oxalaticus* on formate and the capacity of RuBPCase to fix $CO_2$ at various dilution rates in a formate-limited continuous culture were calculated as follows. The rate of $CO_2$ fixation in the culture required to maintain the culture in steady state at dilution rate $D$ is given by $R_r = \frac{D \cdot X \cdot 4}{M}$ *mmol* $CO_2 \cdot h^{-1}$, in which $X$ = bacterial dry weight in the culture (mg/l) and $M$ the "molecular weight" of cell material for which the elementary composition of $C_4H_8O_2N$ was determined experimentally. The potential rate of $CO_2$ fixation based on the activity of RuBPCase is given by

$$R_p = \frac{\text{sp. activity of RuBPCase} \cdot 60 \cdot X \cdot 0.524}{10^6} \text{ mmol } CO_2 \cdot h^{-1}$$

in which the factor 0.524 converts bacterial dry weight ($X$) into protein. This factor indicates that the protein content of cells of *Pseudomonas oxalaticus* was taken as 52.4%. The capacity of RuBPCase is given by $\frac{R_p}{R_r}$.

## Results

In the course of our work on the regulation of mixed substrate utilization in *Pseudomonas oxalaticus* it became apparent that for a better understanding of the rather complex results, the behaviour of the organism during growth on the single substrates was important. Therefore in this section data obtained in oxalate- or formate-limited continuous cultures are considered first, while in the second part results obtained in experiments in which both compounds were present simultaneously will be dealt with.

### *Growth on Single Substrates*

*Pseudomonas oxalaticus* was grown in oxalate- or formate-limited continuous cultures and the capacity of the cells to oxidize these substrates was measured when the culture was in steady state at various dilution rates. In addition, the specific activities of key enzymes of the dissimilatory and assimilatory pathways involved in the metabolism of the two substrates were determined. During growth in the presence of oxalate the capacity of the cells to oxidize oxalate and formate increased with increasing dilution rate in a non-linear fashion (Fig. 1 A). As expected, the specific activity of two of the enzymes involved in the oxidation, namely NAD-FDH and PMS/DCPIP-FDH increased as well. This behaviour was also observed for oxalyl-CoA reductase, a key enzyme of oxalate assimilation; the specific activity of this enzyme increased approximately 2.5-fold as the dilution rate was increased from 0.02 to 0.15 $h^{-1}$.

During growth on formate the capacity of cells to oxidize this substrate also increased with increasing dilution rate (Fig. 1 B) over the range 0.02 to 0.10 $h^{-1}$. Although there was hardly any increase in $Q_{O_2}$ formate above this dilution rate, the specific activity of the two enzymes involved in formate oxidation continued to show a small increase. The specific activity of RuBPCase also increased with dilution rate, but this

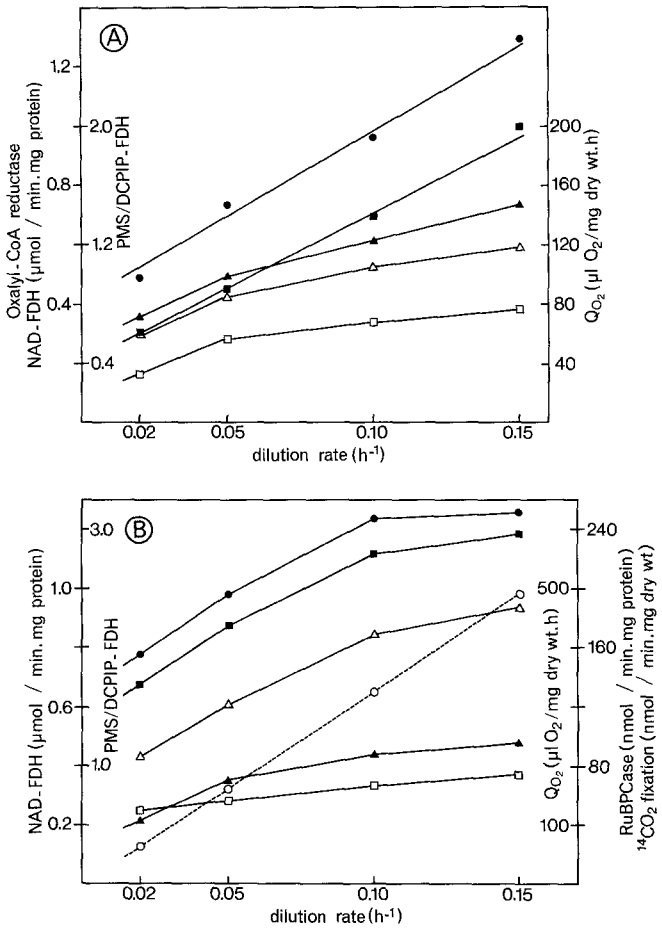


**Fig. 1 A and B.** Relationship between enzyme activity and dilution rate of *Pseudomonas oxalaticus* OX1 during growth on 100 mM oxalate (**A**) and 100 mM formate (**B**). **A** ● Oxalyl-CoA reductase; ■ PMS/DCPIP-FDH; ▲ $Q_{O_2}$ oxalate; △ $Q_{O_2}$ formate; □ NAD-FDH. **B** ● $Q_{O_2}$ formate; ■ RuBPCase (measured); ○ RuBPCase (calculated); △ PMS/DCPIP-FDH; ▲ NAD-FDH; □ $^{14}CO_2$ fixation

increase was only 74% as the dilution rate increased from 0.02 to 0.15 $h^{-1}$. It appeared that even at the highest dilution rate tested the activity of this enzyme is in excess of that required to explain the growth rate. At decreasing dilution rates the overcapacity of this key enzyme of the Calvin cycle increased even further; at a dilution rate of 0.02 $h^{-1}$ it had an activity which was five times greater than that required for the culture to maintain in steady state. The $^{14}CO_2$ fixation rate, measured in washed cell suspensions with excess formate as the energy source, also increased with dilution rate. This rate of $^{14}CO_2$ incorporation is the net effect of the oxidation of formate to provide reducing power, the level of the rate-limiting enzyme(s) in the Calvin cycle and the change in specific activity of the radioactive $CO_2$ in the test mixture as formate is oxidized to $CO_2$. At low dilution rates the difference between RuBPCase activity and the $^{14}CO_2$ fixation rate is mainly due to the fact that the RuBPCase activity is expressed per mg of protein and the $^{14}CO_2$ fixation rate per mg of dry weight, indicating that RuBPCase is probably limiting the rate of $^{14}CO_2$ fixation. At higher dilution rates the ratio of RuBPCase activity/$^{14}CO_2$ fixation rate slightly increased. This is presumably due to a slight increase in dilution of the radioactive label caused by an increasing rate of production of non-labeled $CO_2$ from formate oxidation.

At all dilution rates tested the NAD-FDH present in the cytoplasm and the membrane-bound PMS/DCPIP-linked FDH (Dijkhuizen et al., 1978) displayed higher activities during growth on formate than on oxalate. The same applies to the maximum capacity of whole cells to oxidize formate. After this work was completed, Müller et al. (1978) reported that the soluble NAD-FDH of *P. oxalaticus* also reacts with the redox-dyes PMS and DCPIP. No correction for this effect was made in the present work, so that an unknown part of the activity of the PMS/DCPIP-linked FDH reported in this paper may be due to NAD-FDH. Further details on the membrane-bound PMS/DCPIP-linked FDH will be published elsewhere. The relationships between bacterial dry weight produced during growth on 200 mM ($S_R$) oxalate or 200 mM ($S_R$) formate and dilution rate have been published before (Dijkhuizen et al., 1977b). Essentially the same molar growth yields were obtained during growth on 100 mM oxalate or formate (this paper). The dry weight values, uncorrected for ash content, during growth on oxalate are shown in Fig. 3 (at 0 mM formate). During growth on formate the values were (mg/l): 270 ($D$ = 0.02 $h^{-1}$), 335 ($D$ = 0.05 $h^{-1}$), 364 ($D$ = 0.10 $h^{-1}$) and 365 ($D$ = 0.15 $h^{-1}$). Thus at all dilution rates the dry weight produced during growth on oxalate is approximately 10% higher than during growth on formate.

### *Growth on Mixtures of Oxalate and Formate*

The effect of increasing concentrations of oxalate in the medium reservoir of a formate-limited continuous culture ($S_R$ formate = 100 mM) of *P. oxalaticus* growing at a dilution rate of 0.10 $h^{-1}$ is shown in Fig. 2. The various measurements were made after the culture had reached a steady state. At all ratios of oxalate and formate in the feed, the residual substrate concentration in the culture was undetectably low, indicating that both substrates were utilized to (almost) completion. Increasing amounts of oxalate in the reservoir resulted in the progressive increase in activity of the enzymes of oxalate metabolism, as represented by oxalyl-CoA reductase, the key enzyme in the glycerate pathway. At an oxalate concentration in the mixture of 100 mM, this enzyme reached the same activity as

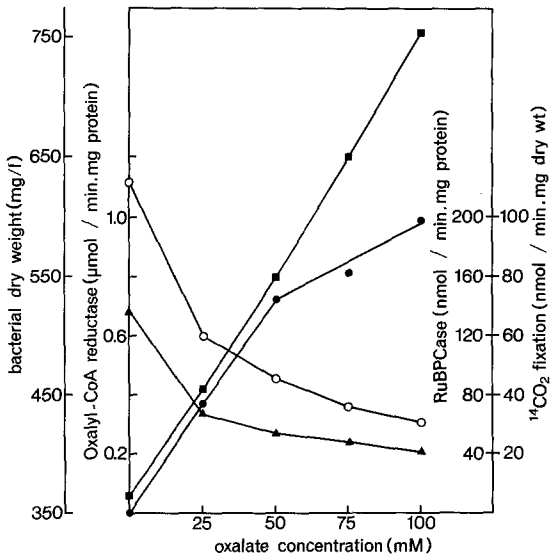


**Fig. 2.** Effect of increasing concentrations of oxalate (0 – 100 mM) in the reservoir of a formate-limited ($S_R$ = 100 mM) continuous culture of *Pseudomonas oxalaticus* OX1 at $D$ = 0.10 $h^{-1}$ on a number of steady state culture parameters. ■ Bacterial dry weight; ● Oxalyl-CoA reductase; ○ RuBPCase; ▲ $^{14}CO_2$ fixation

during growth on 100 mM oxalate alone at the same dilution rate (Fig. 1A). The same held for the $Q_{O_2}$ oxalate. With increasing oxalate concentrations, the synthesis of RuBPCase and the $^{14}CO_2$ fixation rate became progressively repressed. Thus, unlike the situation in batch culture, where formate prevented the utilization of oxalate and caused diauxic growth (Dijkhuizen et al., 1978), in a formate plus oxalate-limited continuous culture oxalate is able to act as a true "heterotrophic" substrate and repress the synthesis of the autotrophic $CO_2$ fixing system.

Even at the highest concentration of oxalate in the reservoir the remaining activity of RuBPCase is such that the same amount of cell material may be synthesized from $CO_2$ during growth on the mixture as during growth on formate alone (Table 1). However, the overcapacity that the enzyme displays during growth on formate alone gradually disappeared as the concentration of oxalate in the mixture increased. The data (Fig. 2) suggest that the potential of the cells to fix $CO_2$ is in fact used during growth on the mixture so that at all ratios of oxalate and formate in the feed both heterotrophic and autotrophic carbon assimilation pathways are functioning simultaneously. Moreover, it appears that the flow of carbon over neither of the two pathways is influenced by the presence of the other substrate in the mixture. This is indicated by the bacterial dry weight produced, which is almost exactly the sum of the dry weight obtained during growth on oxalate and formate separately. Thus, although oxalate is able to repress the synthesis of RuBPCase, under the

**Table 1.** The capacity of RuBPCase to fix $CO_2$ at various concentrations of oxalate in the reservoir of a formate-limited culture of *Pseudomonas oxalaticus* at a dilution rate of 0.10 $h^{-1}$

| Oxalate concentration (mM) | 0 | 25 | 50 | 75 | 100 |
|---|---|---|---|---|---|
| $R_p$: potential rate of $CO_2$ fixation | 2.52 | 1.70 | 1.53 | 1.45 | 1.41 |
| $R_r$: required rate of $CO_2$ fixation[a] | 1.43 | 1.43 | 1.43 | 1.43 | 1.43 |
| $R_p/R_r$: capacity of RuBPCase | 1.76 | 1.19 | 1.07 | 1.01 | 0.99 |

[a] The required rate of $CO_2$ fixation was calculated on the assumption that the same amount of cell material was synthesized from $CO_2$ during growth on the mixture as during growth of the organism on formate alone

experimental conditions of Fig. 2 the remaining activity of this enzyme is sufficiently high to enable the organism to continue to fix $CO_2$ as if oxalate were not present. It is therefore concluded that both substrates are metabolized independently.

As the oxalate concentration was increased, the specific activities of the NAD-FDH, PMS/DCPIP-linked FDH and the maximum capacity to oxidize formate ($Q_{O_2}$ formate) decreased in a parallel fashion and to the same degree. However, since the dry weight of the culture increased with the oxalate concentration, each of these related parameters when expressed per litre culture showed a similar constant capacity to degrade formate (data not shown).

The results obtained in the reverse experiment, i.e. the effect of increasing concentrations of the "autotrophic" substrate formate in the reservoir of an oxalate-limited continuous culture of *P. oxalaticus* are shown in Fig. 3A – D. At each dilution rate tested both substrates were utilized completely irrespective of the ratios of oxalate and formate in the feed and sequential utilization of the substrates was not observed. This was contrary to the results obtained in batch culture experiments (Dijkhuizen et al., 1978). With increasing amounts of formate in the feed, the specific activity of RuBPCase and the capacity to fix $CO_2$ increased in a linear fashion at all dilution rates. The lowest concentration of formate which resulted in RuBPCase synthesis and also the increment of the RuBPCase activity with increasing formate concentrations was dependent on the dilution rate. For instance, at a dilution rate of 0.10 $h^{-1}$ (Fig. 3C) no activity of RuBPCase was detected in the culture below a formate concentration of 43 mM. Nevertheless, at this formate concentration in the feed a 40% increase in the dry weight of the culture was observed. Because RuBPCase activity was absent over this range of formate concentrations, oxalate was the only available carbon

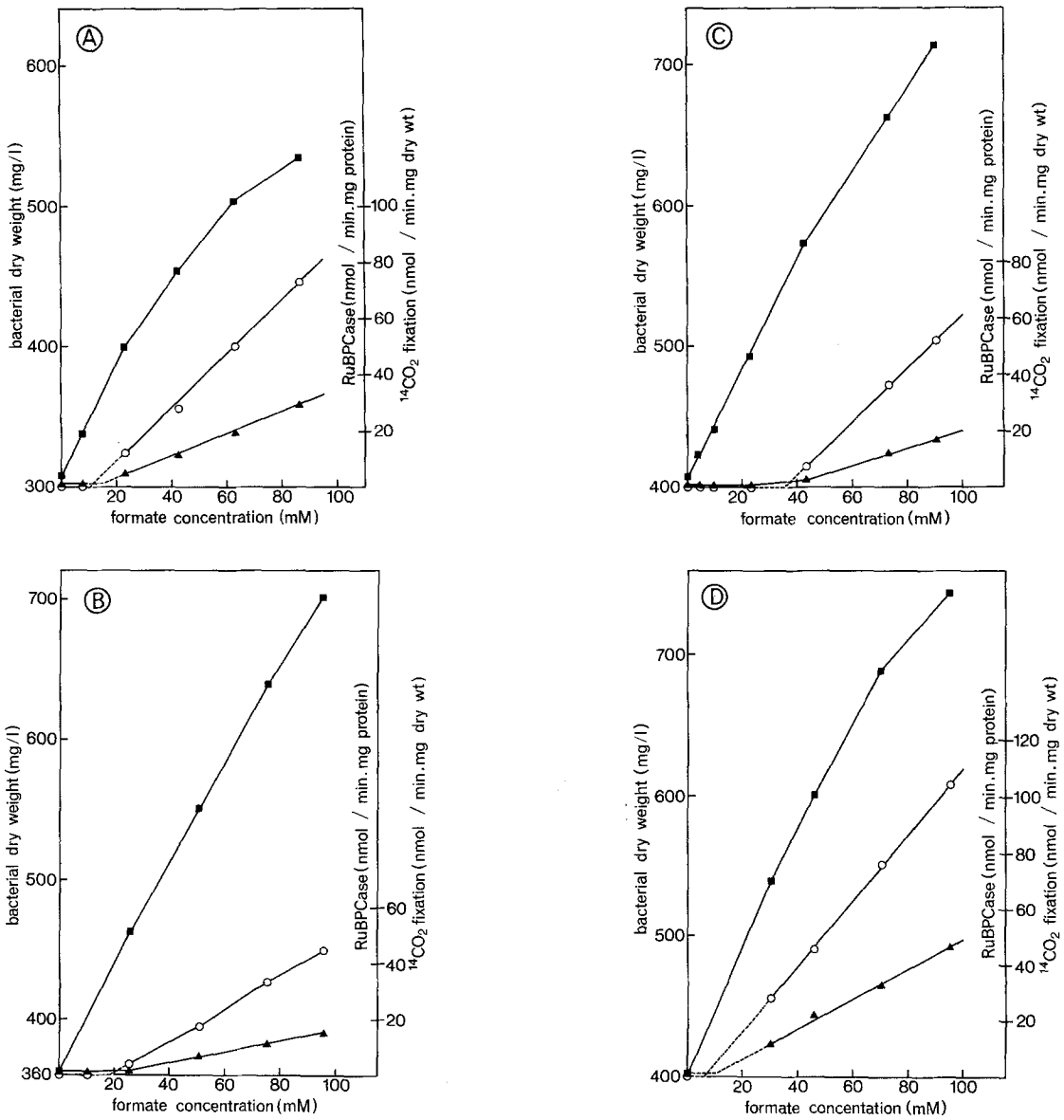


**Fig. 3A–D.** Effect of increasing concentrations of formate (0–100 mM) in the reservoir of an oxalate-limited ($S_R = 100$ mM) continuous culture of *Pseudomonas oxalaticus* OX1 on a number of steady state culture parameters. **A** $D = 0.02\,h^{-1}$; **B** $D = 0.05\,h^{-1}$; **C** $D = 0.10\,h^{-1}$; **D** $D = 0.15\,h^{-1}$. Symbols as in Fig. 2

source and therefore oxalate-carbon must have been redistributed over the assimilatory and dissimilatory pathways at the level of oxalyl-CoA. More oxalate was assimilated and less was dissimilated at increasing formate concentrations, when oxalate was progressively (although only partly) replaced by formate as the energy source. This redistribution of oxalate carbon was not reflected in a change in the specific activity of oxalyl-CoA reductase or the $Q_{O_2}$ oxalate. Both parameters remained constant when formate was added to the medium reservoir (compare Fig. 1A). Similar results were obtained at the three other dilution rates tested, although at these dilution rates the redistribution of oxalate-carbon was not so obvious because of the early appearance of the autotrophic $CO_2$ fixing system at low concentrations of formate in the reservoir (Fig. 3A, B, D).

In the experiments in which oxalate was added to the reservoir of a formate-limited culture (Fig. 2) it was shown that, although oxalate was able to repress the synthesis of RuBPCase, the remaining activity of this enzyme was sufficient to support the conclusion that the rate of autotrophic $CO_2$ fixation by the cells was independent of the presence of oxalate. From the experiments presented in Fig. 3 it appears that this is not always the case during growth of *P. oxalaticus* on

**Table 2.** The capacity of RuBPCase to fix $CO_2$ at various concentrations of formate in the reservoir of an oxalate-limited culture of *Pseudomonas oxalaticus* at different dilution rates

| Dilution rate ($h^{-1}$) | Formate concentration (mM) | 25 | 50 | 75 | 100 |
|---|---|---|---|---|---|
| 0.02 | $R_p$: potential rate of $CO_2$ fixation | 0.18 | 0.56 | 1.02 | 1.49 |
| | $R_r$: required rate of $CO_2$ fixation[a] | 0.05 | 0.11 | 0.16 | 0.21 |
| | $R_p/R_r$: capacity of RuBPCase | 3.60 | 5.09 | 6.38 | 7.10 |
| 0.05 | $R_p$: potential rate of $CO_2$ fixation | 0.05 | 0.31 | 0.66 | 1.07 |
| | $R_r$: required rate of $CO_2$ fixation[a] | 0.16 | 0.33 | 0.49 | 0.66 |
| | $R_p/R_r$: capacity of RuBPCase | 0.31 | 0.94 | 1.35 | 1.62 |
| 0.10 | $R_p$: potential rate of $CO_2$ fixation | – | 0.25 | 0.78 | 1.42 |
| | $R_r$: required rate of $CO_2$ fixation[a] | 0.36 | 0.71 | 1.07 | 1.43 |
| | $R_p/R_r$: capacity of RuBPCase | – | 0.35 | 0.73 | 0.99 |
| 0.15 | $R_p$: potential rate of $CO_2$ fixation | 0.36 | 1.00 | 1.77 | 2.60 |
| | $R_r$: required rate of $CO_2$ fixation[a] | 0.54 | 1.07 | 1.61 | 2.15 |
| | $R_p/R_r$: capacity of RuBPCase | 0.67 | 0.94 | 1.10 | 1.21 |

[a] The required rate of $CO_2$ fixation was calculated on the assumption that the same amount of cell material was synthesized from $CO_2$ during growth on the mixture as during growth of the organism on formate alone

mixtures of formate and oxalate. Not only have conditions been found where the presence of formate in the mixture did not lead to the synthesis of RuBPCase in the cells ($D = 0.10\,h^{-1}$, 25 mM formate), there are also instances (e.g. $D = 0.05\,h^{-1}$, at formate concentrations below 50 mM; $D = 0.10\,h^{-1}$, below formate concentrations of 100 mM and $D = 0.15\,h^{-1}$, below formate concentrations of 50 mM) where the specific activity of the enzyme is too low to meet the required rate of $CO_2$ fixation (Table 2). The data (Fig. 3) indicate, however, that if RuBPCase is present in the cells during growth of the organism on the mixture, the Calvin cycle does contribute to the in vivo carbon assimilation to an extent which is mainly governed by the activity of the enzyme. This suggestion is supported by the following observations. At all dilution rates tested (Fig. 3A – D) the dry weight of the cultures increased with increasing formate concentrations. This increase was linear, until at a certain formate concentration in the feed it started to deviate from linearity. This always coincided with the appearance of RuBPCase and $^{14}CO_2$ fixation in the culture and the deviation from linearity became more apparent as the level of activity of the autotrophic enzyme increased. This decrease in growth yield is due to an increasing contribution of the energetically more expensive autotrophic $CO_2$ fixation to the biosynthesis of cell material (see also DeCicco and Stukus, 1968; Rittenberg and Goodman, 1969). In the present experiments the effect is not as evident as during growth of the organism on mixtures of acetate and formate (Dijkhuizen and Harder, 1979), because the molar growth yields of *P. oxalaticus* on oxalate and formate are not very different (Dijkhuizen et al., 1977b). Nevertheless, Fig. 3 shows that during growth on the mixture the molar growth yield observed was equal to, or slightly higher than, that measured during growth on oxalate alone under those conditions where RuBPCase activity was absent. Under conditions where RuBPCase activity was present, the growth yields had decreased to values in between those measured during growth on oxalate and formate separately, thus reflecting the contribution of autotrophic $CO_2$ fixation to the biosynthesis of cell material. It is therefore suggested that during growth of *P. oxalaticus* on mixtures of oxalate and formate, the contribution of autotrophic $CO_2$ fixation to the biosynthesis of cell material is controlled via the synthesis of RuBPCase (and possibly phosphoribulokinase) rather than by control over the activity of existing enzyme(s).

As shown in Fig. 1A and 1B, the activities of the formate-oxidizing enzymes are higher during growth on formate than during growth on oxalate. Upon the addition of formate to the reservoir of an oxalate-limited continuous culture the specific activities of these enzymes increased in a parallel manner. During growth on the mixture of 100 mM oxalate and 100 mM formate, these enzymes reached levels close to those measured during growth on formate alone, but per litre of culture the capacity to oxidize formate was about twice as high. This behaviour deviates from the results obtained in the reverse experiment when oxalate was added to the reservoir of a formate-limited culture (see above).

The specific activities of RuBPCase, presented in Fig. 3A – D, have also been plotted against the dilution rate for four different mixtures of formate and oxalate in the reservoir (Fig. 4). For each of these mixtures there are two regions of derepression of RuBPCase synthesis, namely at low and at high dilution rates. This relationship between RuBPCase activity and dilution rate was confirmed in control experiments in which fixed

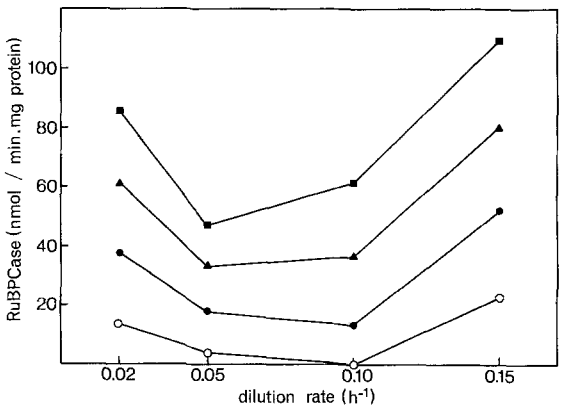


**Fig.4.** Relationship between RuBPCase activity and dilution rate of *Pseudomonas oxalaticus* OX1 during growth on mixtures of oxalate ($S_R$ = 100 mM) and various concentrations of formate in the reservoir. Formate concentration: ○ 25 mM; ● 50 mM; ▲ 75 mM; ■ 100 mM

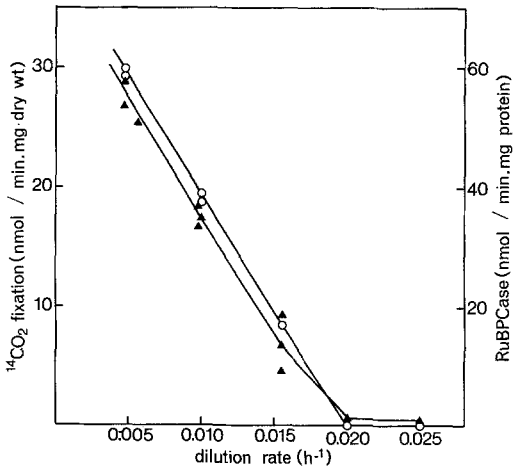


**Fig.5.** Relationship between RuBPCase activity, $^{14}CO_2$ fixation rate and dilution rate of *Pseudomonas oxalaticus* OX1 during growth on oxalate ($S_R$ = 200 mM) alone. Symbols as in Fig.2

ratios of oxalate and formate were used in the reservoir while the dilution rate was changed.

Derepression of RuBPCase synthesis and $^{14}CO_2$ fixation was also observed during growth of *P. oxalaticus* in carbon- and energy-limited continuous cultures with the "heterotrophic" substrate oxalate alone (Fig. 5). When the dilution rate was decreased below 0.02 $h^{-1}$, i.e. below approximately 10% of the maximum specific growth rate on oxalate, a rapid appearance of the autotrophic $CO_2$ fixing system was observed. The highest specific activity of RuBPCase detected at $D$ = 0.005 $h^{-1}$ (60 nmol/min · mg protein) was about 50% of the value found during growth of the organism at this dilution rate on formate. In this experiment the viability of the culture decreased from 93% ($D$ = 0.02 $h^{-1}$) to 85.7% ($D$ = 0.005 $h^{-1}$). No correction for this slight decrease of viability has been made in Fig. 5. Derepression of RuBPCase synthesis was not observed at low dilution rates in glyoxylate-, glycerol-, malonate- or acetate-limited continuous cultures. Derepression of autotrophic $CO_2$ fixation was also reported by Slater and Morris (1973), after depriving photoheterotrophically grown cultures of *Rhodospirillum rubrum* of malate.

## Discussion

As in many facultative autotrophs, the synthesis of RuBPCase in *P. oxalaticus* is totally repressed when the organism is grown in batch culture on a "heterotrophic" substrate such as acetate, lactate, glycollate or oxalate. Repression is also evident during growth of the organism in batch culture on mixtures of these substrates and formate with one exception, namely oxalate; in the latter case formate prevented the utilization of oxalate and caused diauxic growth (Dijkhuizen et al., 1978). However, when oxalate was added to the reservoir of a formate-limited continuous culture of *P. oxalaticus*, strong repression of the synthesis of RuBPCase was observed (Fig. 2), indicating that under these conditions oxalate behaved as a typical "heterotrophic" substrate in a way similar to acetate (Dijkhuizen and Harder, 1979). Apparently a relatively high concentration of formate is required to prevent the metabolism of oxalate. However, when the formate concentration in the culture drops below a certain value and the inhibition of oxalate metabolism is lifted, its metabolism may generate one or more intermediates which are able to repress the synthesis of RuBPCase in a way similar to that observed for acetate. More extensive discussions of the general implications of mixed substrate utilization in batch and continuous culture have been given elsewhere (Harder and Dijkhuizen, 1976).

When formate was added to the reservoir of an oxalate-limited culture of *P. oxalaticus*, a rather complex behaviour was observed (Fig. 3A–D). Below a certain critical formate concentration which was dependent on the dilution rate, formate was only utilized as an ancillary energy source for oxalate assimilation. This allowed an increased flow of oxalate carbon over the assimilation pathway resulting in an increase in the dry weight of the culture. This increase of dry weight was not reflected by an increase in the specific activity of oxalyl-CoA reductase; the activity of this key enzyme of oxalate assimilation was very high (> 0.5 μmol/mg protein · min) and was only dependent on the dilution rate (Fig. 1A). The observed increase in

dry weight of the culture can only be explained by a redistribution of oxalate carbon over the branch point of oxalyl-CoA utilization. The two enzymes competing for this intermediate, the dissimilatory oxalyl-CoA decarboxylase and the assimilatory oxalyl-CoA reductase both have a similar high $V_{max}$ and a $K_m$ for oxalyl-CoA of 1 mM and 0.2 mM, respectively (Quayle, 1969; Laanbroek, unpublished). The utilization of oxalyl-CoA for either dissimilatory or assimilatory purposes is most probably controlled by the relative levels of reduced and oxidized pyridine nucleotides. Knight et al. (1978) have already shown in substrate transition experiments that the addition of formate to an oxalate-grown culture of *P. oxalaticus* resulted in a 3-fold increase in the ratio NADH/total $NAD^+$ and a 1.5-fold increase in NADPH/total $NADP^+$ ratio. Similarly, the addition of formate to the reservoir of an oxalate-limited culture may result in an increased rate of NADH production from formate oxidation. Due to transhydrogenase activity, which in *P. oxalaticus* is presumably soluble and non-energy linked (Quayle et al., 1961), the rate of NADPH production from NADH and $NADP^+$ may therefore also increase and enhance the in vivo turnover rate of oxalyl-CoA to glyoxylate, thereby enhancing the flow of oxalate carbon towards assimilation. Presumably, with increasing formate concentrations in the medium a situation will occur (depending on the dilution rate) in which the rate of oxalyl-CoA production limits its own conversion. We suggest that one of the enzymes involved in the production of oxalyl-CoA from oxalate is in fact rate-limiting and therefore also limits the maximum specific growth rate of *P. oxalaticus* on oxalate. If this suggestion is correct, then the increased rate of ATP and NADH production from the oxidation of formate will drain the pool of intermediates for biosynthesis which in turn results in derepression of RuBPCase and autotrophic $CO_2$ fixation. Once derepression of RuBPCase occurred in response to the presence of sufficient formate in the medium, both autotrophic and heterotrophic carbon assimilation pathways functioned concomitantly as indicated by the bacterial dry weight produced. It is therefore concluded that in *P. oxalaticus*, the contribution of autotrophic $CO_2$ fixation to the biosynthesis of cell material is controlled via the synthesis of RuBPCase (and possibly phosphoribulokinase).

The relationship between the observed activity of RuBPCase in the cells and the dilution rate during growth of *P. oxalaticus* on mixtures of oxalate and formate is also rather complex (Fig. 4). Irrespective of the formate concentration in the feed a progressive derepression of the synthesis of RuBPCase is observed at dilution rates below $0.05\,h^{-1}$. This is probably caused by the fact that the intracellular concentration of the repressor molecule(s) involved in exerting repression decreases at the lower dilution rates, parallel to the decreasing concentration of the growth-limiting carbon- and energy source in the culture (Herbert et al., 1956). Similar results have been reported for photoheterotrophic growth of *Rhodospirillum rubrum* in a malate-limited chemostat (Slater and Morris, 1973). The derepression observed at high dilution rates (above $0.10\,h^{-1}$) may be explained as follows. Initially, with increasing dilution rates, repression became more severe but starting at a certain dilution rate, the rate of oxalyl-CoA utilization in the cells may have become faster than the rate of oxalyl-CoA production. This may have resulted in a decrease in the concentration of internal metabolites for biosynthesis, including that of the repressor molecule(s). During growth on oxalate alone this decrease was not sufficient to cause derepression of RuBPCase synthesis; this only became apparent during growth in the presence of formate when formate oxidation added an additional drain on the pool size of metabolic intermediates. The observation that at higher ratios of formate to oxalate in the feed, the dilution rates at which RuBPCase synthesis became derepressed were lower (Fig. 4) is in complete agreement with this suggestion.

Although our results do not totally exclude the possibility that the synthesis of Calvin cycle enzymes in *P. oxalaticus* is regulated by induction modified by catabolite repression (Blackmore and Quayle, 1968), they strongly suggest that control is exerted through a repression/derepression mechanism. The reasons for this conclusion can be summarized as follows. At a dilution rate of $0.10\,h^{-1}$, addition of formate to the reservoir of an oxalate-limited continuous culture did not result in RuBPCase synthesis if the formate concentration in the mixture was below 43 mM (Fig. 3C). If formate, or a metabolite produced from formate were the inducer of the autotrophic enzymes, then this behaviour would not be consistent with that expected of such a regulatory system. Moreover, the derepression of the Calvin cycle enzymes observed at low dilution rates in an oxalate-limited culture (Fig. 5) undoubtedly showed that even in the absence of extracellular formate, under appropriate conditions, the synthesis of RuBPCase and autotrophic $CO_2$ fixation may be initiated. This was also apparent in a mutant of *P. oxalaticus* which was blocked in the glycerate pathway (glyoxylate carboligase negative) and able to grow autotrophically on oxalate (Dijkhuizen, unpublished). A situation in which very low concentrations of intermediary metabolites occur in the cells seems to be required for derepression of Calvin cycle enzymes. This indicates that the repressor of autotrophic $CO_2$ fixation may be found in this pool of metabolites. Although derepression of RuBPCase

was observed during growth on oxalate alone, at low dilution rates and in the mutant, none of the other substrates tested showed this behaviour. This may be caused by the fact that the main flow of oxalate carbon is via formyl-CoA directly to carbon dioxide (Dijkhuizen et al., 1977b), and not via the central pathways of intermediary metabolism.

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